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# The *Melampsora lini AvrL567* Avirulence Genes Are Expressed in Haustoria and Their Products Are Recognized inside Plant Cells

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**The *Linum usitatissimum* (flax) *L* gene alleles, which encode nucleotide binding site–Leu rich repeat class intracellular receptor proteins, confer resistance against the *Melampsora lini* (flax rust) fungus. At least 11 different *L* resistance specificities are known, and the corresponding avirulence genes in *M. lini* map to eight independent loci, some of which are complex and encode multiple specificities. We identified an *M. lini* cDNA marker that cosegregates in an F2 rust family with a complex locus determining avirulence on the *L5*, *L6*, and *L7* resistance genes. Two related avirulence gene candidates, designated *AvrL567-A* and *AvrL567-B*, were identified in a genomic DNA contig from the avirulence allele, whereas the corresponding virulence allele contained a single copy of a related gene, *AvrL567-C*. *Agrobacterium tumefaciens*–mediated transient expression of the mature AvrL567-A or AvrL567-B (but not AvrL567-C) proteins as intracellular products in *L. usitatissimum* and *Nicotiana tabacum* (tobacco) induced a hypersensitive response–like necrosis that was dependent on coexpression of the *L5*, *L6*, or *L7* resistance gene. An F1 seedling lethal or stunted growth phenotype also was observed when transgenic *L. usitatissimum* plants expressing AvrL567-A or AvrL567-B (but not AvrL567-C) were crossed to resistant lines containing *L5*, *L6*, or *L7*. The *AvrL567* genes are expressed in rust haustoria and encode 127 amino acid secreted proteins. Intracellular recognition of these rust avirulence proteins implies that they are delivered into host cells across the plant membrane. Differences in the three AvrL567 protein sequences result from diversifying selection, which is consistent with a coevolutionary arms race.**

## INTRODUCTION

The gene-for-gene model of plant disease resistance (Flor, 1971) predicts that plant resistance (*R*) gene products recognize the direct or indirect product of pathogen avirulence (*Avr*) genes. This recognition event activates host defense responses, which include a localized host cell death or hypersensitive response (HR). The majority of *R* genes identified to date encode proteins containing a predicted nucleotide binding site (NBS) followed by a series of Leu-rich repeats (LRR) at their C termini and are predicted to be intracellular (Dangl and Jones, 2001; Staskawicz et al., 2001). Different genes in this group confer host resistance to viruses, bacteria, oomycetes, fungi, nematodes, and sucking insects. NBS-LRR resistance proteins also may contain N-terminal TIR (Toll and Interleukin-1 receptor homology) or coiled coil domains. The highly variable LRR domains are implicated in controlling recognition specificity (Ellis et al., 1999; Jia et al., 2000; Dodds et al., 2001b), whereas intramolecular interactions involving the TIR-NBS or coiled coil NBS regions may control propagation of the perceived signal (Moffet et al., 2002; Hwang and Williamson, 2003). A second major class of *R* genes encode membrane-bound receptor proteins with an extracellular LRR domain, sometimes coupled to an intracellular kinase signaling domain (Song et al., 1995; Dixon et al., 1996, 1998; Parniske et al., 1997).

In contrast with the conserved structure of most R proteins, pathogen Avr proteins are extremely varied, reflecting the diverse array of pathogen molecules to which plants are exposed and which could be used as recognition targets. For instance, bacterial Avr products are members of a suite of disease effector proteins that are delivered directly into the plant cytoplasm via a type III secretion system (Lahaye and Bonas, 2001; Casper-Lindley et al., 2002). The bacterial type III secretion system is encoded by the hypersensitive response and pathogenicity (*Hrp*) genes and is essential for infection because *hrp* mutants are no longer pathogenic on their host plants. Whereas the biochemical functions of most bacterial effector proteins are unknown, some are targeted to the host nucleus and may act as transcription factors (Lahaye and Bonas, 2001; Deslandes et al., 2003). Other bacterial effectors are proteases (Orth et al., 2000), and some of these are known to cleave specific host proteins (Axtell et al., 2003; Shao et al., 2003).

Colocalization of corresponding R and Avr products is probably a prerequisite for recognition. Resistance proteins corresponding to bacterial Avr products targeted to the host cytoplasm all belong to the intracellular NBS-LRR class (Staskawicz et al., 2001), and resistance proteins against intracellular

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viral pathogens are also NBS-LRR proteins (Whitham et al., 1996; Bendahmane et al., 1999; Cooley et al., 2000). By contrast, the *Lycopersicon esculentum* (tomato) Cf resistance proteins are members of the extracellular receptor family and recognize Avr products secreted by the fungus *Cladosporium fulvum* into the apoplastic space (Dixon et al., 1996, 1998; Parniske et al., 1997). In some cases, recognition involves direct protein–protein interactions between corresponding R and Avr proteins (Jia et al., 2000; Deslandes et al., 2003). However, the RPS2 (resistance to *Pseudomonas syringae*) and RPM1 (resistance to *P. syringae* pv *maculicola*) resistance proteins in Arabidopsis (*Arabidopsis thaliana*) apparently recognize their corresponding Avr products indirectly by detecting changes induced in a host protein that is modified by these Avr products (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003). Nevertheless, all of the components of the RPM1/RPS2 recognition complexes colocalize and are peripherally associated with the plasma membrane (Boyes et al., 1998; Axtell and Staskawicz, 2003). Similarly, the Arabidopsis RRS1 (resistance to *Ralstonia solanacearum*) protein and its corresponding Avr protein, PopP2, also colocalize, in this case to the host cell nucleus (Deslandes et al., 2003).

Rust fungi (order Uredinales) cause disease on many important crop plants and are obligate biotrophs that grow only in living plant tissue. During infection, rusts form haustoria, specialized structures that penetrate the plant cell wall but remain separated from the host cytoplasm by the host cell membrane. One of the primary functions of haustoria seems to be to facilitate nutrient uptake from the host (Hahn and Mendgen, 2001). Haustoria formation also induces structural changes in the host cell, including cytoskeletal rearrangements, nuclear migration, and chromatin condensation (Kobayashi et al., 1994; Heath, 1997), and there is some evidence that compatible rusts can suppress host defense responses and influence host cell metabolism (Mellersh and Heath, 2001; Ayliffe et al., 2002; Voegele and Mendgen, 2003). However, the signals leading to these changes are not known. In resistant plants, haustoria formation also seems to be the trigger for HR induction (Kobayashi et al., 1994; Heath, 1997), suggesting that rust Avr products are detected at these sites.

In *Linum usitatissimum* (flax), at least 30 rust resistance specificities have been distinguished by their recognition of different *Melampsora lini* (flax rust) strains, and these are distributed among five polymorphic loci, *K*, *L*, *M*, *N*, and *P* (Islam and Mayo, 1990). R proteins encoded by genes at the *L*, *M*, *N*, and *P* loci are members of the intracellular NBS-LRR class (Lawrence et al., 1995; Anderson et al., 1997; Dodds et al., 2001a, 2001b). NBS-LRR proteins also confer resistance to rusts in maize (*Zea mays*) (Collins et al., 1999), and similar proteins in Arabidopsis, *Lactuca sativa* (lettuce), and *Hordeum vulgare* (barley) confer resistance to downy mildew and powdery mildew pathogens (Noel et al., 1999; Halterman et al., 2001; Zhou et al., 2001; Shen et al., 2002), which are also haustoria-producing obligate biotrophs. Although *Avr* genes have been described at a genetic level in rusts and mildews (Flor, 1971; Lawrence et al., 1981; Zambino et al., 2000; Pedersen et al., 2002; Rehmany et al., 2003), no Avr products have been identified in these pathogens, so the conundrum of how intracellular R proteins recognize these extracellular pathogens remains unresolved.

In this article, we describe the isolation of three members of a family of avirulence genes from *M. lini* that are recognized by the *L5*, *L6*, and *L7* resistance genes. A cDNA marker cosegregating with the avirulence phenotype in an F2 rust family was used to identify a genomic DNA region from the avirulence locus. We show that in planta expression of avirulence gene candidates encoded at this locus causes *R* gene–dependent cell death that is specific to the *L5*, *L6*, and *L7* genes. The *M. lini* avirulence genes are expressed in haustoria and encode small secreted proteins. Recognition of these proteins occurs when they are expressed inside the plant cell, suggesting that they are delivered into host cells during rust infection.

## RESULTS

### Isolation of a cDNA Marker Cosegregating with an *M. lini* Avirulence Locus

To isolate *Avr* genes from *M. lini*, we undertook a map-based cloning approach using an F2 family of 74 individuals produced by crossing the rust strains C and H and then self-fertilizing the F1 hybrid rust strain CH5 (Figure 1; Lawrence et al., 1981). Avr specificities corresponding to sixteen different resistance genes segregate in this family and map to 10 independent loci. Several markers linked to Avr loci were identified using random amplified polymorphic DNA, amplified fragment length polymorphism, and cDNA amplified fragment length polymorphism techniques (P.N. Dodds, M.A. Ayliffe, G.J. Lawrence, and J.G. Ellis, unpublished results) as well as by mapping in planta–expressed rust cDNA clones as restriction fragment length polymorphisms (RFLPs). For the latter approach, we used suppression subtractive

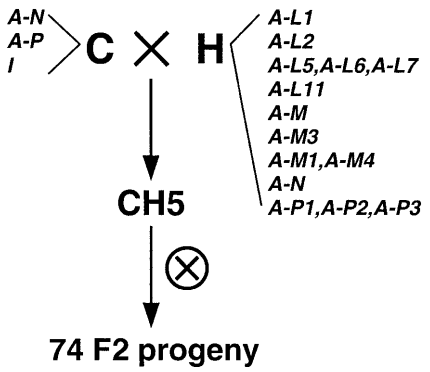


**Figure 1.** An *M. lini* Family Segregating for 16 Avirulence Specificities.

The rust strains C and H were crossed together to produce the F1 rust CH5, which was then selfed to produce 74 F2 progeny (Lawrence et al., 1981). Avirulence specificities segregating in the F2 family are indicated next to the parental rust from which they were inherited. Some avirulence specificities, such as *A-L5*, *A-L6*, and *A-L7*, cosegregate in this family and are listed next to each other. The *A-P* gene from rust C segregates as an alternative allele at the same locus as *A-P1*, *A-P2*, and *A-P3* from rust H. The *A-N* gene was present in both parents but segregates in the F2. An inhibitor gene (*I*) that suppresses resistance reactions triggered by some avirulence genes was inherited from rust C.

hybridization (Diatchenko et al., 1996) to prepare a cDNA library enriched for transcripts present in *L. usitatissimum* leaves infected with rust H but not in uninfected leaves (see Methods). A second subtracted cDNA library was enriched for transcripts more abundant during infection with rust H than with rust C because most of the *Avr* genes segregating in the F2 family were derived from rust H. A total of 181 randomly selected cDNA clones from these libraries were sequenced and represented 112 unique cDNAs. DNA gel blot hybridization confirmed that 80 clones were derived from the rust genome, of which 45 detected RFLPs between the parental rust strains C and H. These markers were scored in the 74 F2 individuals.

One cDNA probe, isolated from the infected versus uninfected cDNA library and designated IU2F2, detected RFLPs that cosegregated with an avirulence locus corresponding to the *L5*, *L6*, and *L7* resistance genes in *L. usitatissimum*. These three avirulence specificities cosegregate in several independent rust families (Lawrence et al., 1981) and have not been separated by mutation (Flor, 1956). In digests with numerous restriction enzymes, the 180-bp IU2F2 cDNA probe consistently detected two hybridizing fragments that cosegregated with the avirulence allele derived from rust strain H (avirulent on *L5*, *L6*, and *L7*), indicating that this allele contains a duplication of this sequence (data not shown). A single restriction fragment hybridizing to IU2F2 segregated at the alternative allele derived from rust strain C (virulent on *L5*, *L6*, and *L7*).

## A Candidate Avirulence Gene Encodes a Small Secreted Protein

Because the IU2F2 marker cosegregated with the avirulence locus, we used this probe to isolate further sequences from this gene and surrounding DNA. A sequence contig of 26.5 kb (Figure 2A) was assembled from several overlapping λ clones isolated from a genomic DNA library prepared from the F1 rust CH5 (Ayliffe et al., 2001). By comparison with the restriction fragment sizes detected by genomic DNA gel blot hybridization, this sequence corresponded to the rust H–derived allele of the IU2F2 RFLP locus. Because the alternative allele was not recovered from the genomic DNA library, the corresponding sequences were amplified by long PCR from an F2 rust strain homozygous for this allele. The restriction map of the resulting 11.5-kb contig was consistent with the IU2F2-detected restriction fragments derived from the virulent rust strain C. An RFLP detected using a probe from the 3′ end of these contigs was separated from the IU2F2 marker and avirulence genes by three recombination events in the F2 family (data not shown). Amplification and sequence analysis of this genomic DNA region from the three recombinants confirmed that the crossovers occurred within 1 kb of the 3′ end of the IU2F2 contig (Figure 2A; data not shown). This region therefore delineated one end of the genetic interval containing the avirulence genes. Although the physical contig did not encompass the other end of this genetic interval, the high frequency of recombination suggested that this region might

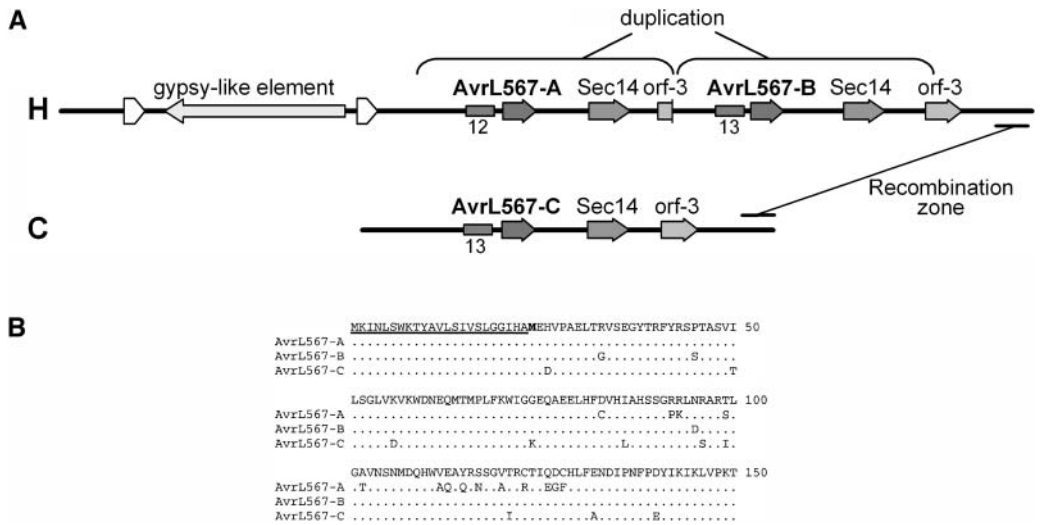


**Figure 2.** Characterization of the *M. lini AvrL567* Locus.

**(A)** Schematic diagram of the *AvrL567* locus. A 26.5-kb region from the avirulence allele (H) contains the *AvrL567-A* and *AvrL567-B* genes, whereas the *AvrL567-C* gene occurs in an 11.5-kb region at the virulence allele (C). A second gene related to yeast *Sec14* (Bankaitis et al., 1989) also occurs in this region along with a third predicted gene (*orf-3*; no similarity to database sequences). The position of a 7.4-kb duplication in the H allele is indicated, as is a 6.3-kb inserted sequence similar to gypsy class retrotransposons, with 174-bp identical direct repeats flanking a long open reading frame encoding a putative polyprotein. A repeat region containing 12 or 13 copies of an 82-bp sequence, which occurs close to the 5′ ends of the *AvrL567* genes, is shown as a closed box. An ~1-kb region in which three recombination events were resolved is indicated at the 3′ end of the contigs.

**(B)** Amino acid sequence alignment of the predicted AvrL567-A, AvrL567-B, and AvrL567-C proteins. Only those amino acids that differ from the consensus (top line) are shown, with identical residues indicated by a dotted line. The signal peptide is underlined, and the first amino acid of the predicted mature protein (Met-24) is shown in bold.

have a low physical distance to map distance ratio, and we were encouraged to examine the sequences for candidate genes.

Three distinct classes of genes were detected in the DNA sequences derived from the IU2F2 locus, each with two copies present at the H allele and a single copy at the C allele (Figure 2A). One of these corresponded to the IU2F2 cDNA clone and appeared to be a likely candidate to encode avirulence because there was a high level of sequence variation between the virulence and avirulence alleles. In planta expression assays (see below) showed that these genes triggered *R* gene–dependent cell death responses with specificity for the *L5*, *L6*, and *L7* resistance genes. We have therefore designated these genes as *AvrL567-A*, *AvrL567-B* (both from the avirulence allele), and *AvrL567-C* (from the virulence allele). Reverse transcriptase (RT)-PCR products amplified from rust-infected leaf RNA indicated that the *AvrL567* transcripts contain 450-bp open reading frames. Two introns of 85 and 215 bp, both within the 5′ untranslated leader, were present in the genomic sequence but were spliced from the cDNA-derived PCR products. The 150 amino acid translations of the *AvrL567* genes include predicted 23–amino acid cleavable secretion signal peptides (PSORT, http://psort.nibb.ac.jp/), suggesting that they encode secreted proteins of 127 amino acids. The amino acid sequences are highly variable, with 27 polymorphic sites in the 127 amino acid mature products (Figure 2B). No related sequences were detected in public databases, and no other recognizable functional motifs were detected.

A second gene family encoded in this region is closely related to the yeast gene *Sec14* (Bankaitis et al., 1989), which encodes a phosphatidylinositol-phosphatidylcholine transfer protein involved in membrane fusion (Lopez et al., 1994). A third gene (*orf-3*) with no related sequences in the public sequence databases was predicted based on the presence of two open reading frames of 333 and 342 bp separated by a short putative intron (87 bp). However, we did not consider these genes to be likely candidates to encode the avirulence specificities that cosegregate with this locus because there was very little sequence variation between homologs encoded at the two alleles. The three *Sec14* homologs are almost identical, with only a single conservative amino acid substitution (glutamate to aspartate) in their predicted products, whereas the *orf-3* homologs are identical except for the truncation of one copy at a duplication boundary in the H allele (Figure 2A). In addition, *Sec14* and *orf-3* transcripts were detected at similar levels in leaves infected with either virulent or avirulent rust strains (data not shown).

### Transient Expression of *AvrL567* Genes in *L. usitatissimum* Causes Resistance Gene–Dependent Cell Death

Because no transformation procedure exists for rust fungi, it was not possible to directly test the avirulence phenotype conferred by the *AvrL567* genes in transgenic rust. However, one of the primary outcomes triggered by R-Avr recognition is the HR, and *Agrobacterium tumefaciens*–mediated transient expression of corresponding *R* and *Avr* genes in plants has been shown to induce HR-like cell death (Scofield et al., 1996; Tang et al., 1996; van den Ackervecken et al., 1996; Bendahmane et al., 1999; Erickson et al., 1999; Tai et al., 1999; van der Hoorn et al., 2000). We therefore generated T-DNA constructs encoding the 127–amino acid mature AvrL567 proteins beginning at Met-24 (without the 23–amino acid signal peptides) driven by the 35S promoter of *Cauliflower mosaic virus* for transient expression assays. *A. tumefaciens* strains containing the AvrL567-A$^{127}$ construct or an *L6* gene construct were infiltrated into leaves of rust-susceptible *L. usitatissimum* var Hoshangabad (Figure 3A). *L. usitatissimum* leaves infiltrated with an *A. tumefaciens* strain containing an empty binary vector, or with the L6 or AvrL567-A$^{127}$ constructs alone, did not cause any visible effects. However, coexpression of AvrL567-A$^{127}$ and L6 in *A. tumefaciens*–infiltrated leaves induced a necrotic response, indicating that the AvrL567-A$^{127}$ product is recognized by L6.

To further test the recognition specificity of the avirulence genes, *A. tumefaciens* cultures containing T-DNA constructs encoding AvrL567-A$^{127}$, AvrL567-B$^{127}$, or AvrL567-C$^{127}$ were infiltrated into leaves of near-isogenic *L. usitatissimum* lines carrying different *L* locus rust resistance alleles (Figure 3B). Expression of AvrL567-A$^{127}$ induced a strong necrotic response in *L5* and *L6* plants and a weak response in *L7* plants, whereas expression of AvrL567-B$^{127}$ induced a strong response in *L5* plants, a weak response in *L6* plants, and no response on *L7*. Thus, the *AvrL567-A* and *AvrL567-B* genes are sufficient to account for the three recognition specificities that cosegregate at this avirulence locus. No responses were observed when these constructs were tested in *L. usitatissimum* lines containing *L*, *L1*, *L2*, *L3*, *L4*, *L8*, *L9*, or *L10* (Figure 3, Table 1), indicating that recognition was specific to the *L5*, *L6*, and *L7* resistance genes. Importantly, transient expression of AvrL567-C$^{127}$, encoded by the homologous gene from the virulent parental rust strain C, did not induce necrosis in any *L. usitatissimum* line (Figure 3B, Table 1). RNA gel blot analysis confirmed that the AvrL567$^{127}$ constructs were each expressed at similar levels in *A. tumefaciens*–infiltrated leaves (data not shown).

Infiltration with *A. tumefaciens* strains containing the AvrL567-A$^{127}$ or AvrL567-B$^{127}$ T-DNA construct, but no Ti plasmid to enable T-DNA transfer, did not induce necrosis, and AvrL567 transcripts were not detected by RNA gel blot analysis (data not shown). Thus, the necrotic reactions were dependent on production of the AvrL567$^{127}$ proteins in transformed plant cells rather than recognition of *A. tumefaciens*–expressed proteins. Infiltration with *A. tumefaciens* strains containing T-DNA constructs encoding the full-length AvrL567$^{150}$ proteins (including the secretion signal) induced much weaker reactions than the truncated versions, although they maintained a similar relative specificity (data not shown). These responses took longer to develop (10 to 12 d compared with 4 to 5 d) and were characterized by chlorosis rather than necrosis.

### Transient Coexpression of L6 and AvrL567 Induces Cell Death in *Nicotiana tabacum*

To determine whether coexpression of the *L6* and *AvrL567* genes could induce an HR in other species, these genes were transiently expressed in *Nicotiana tabacum* (tobacco). *A. tumefaciens*–mediated transient coexpression of AvrL567-A$^{127}$ and L6 produced a necrotic response in *N. tabacum* (Figure 3C).

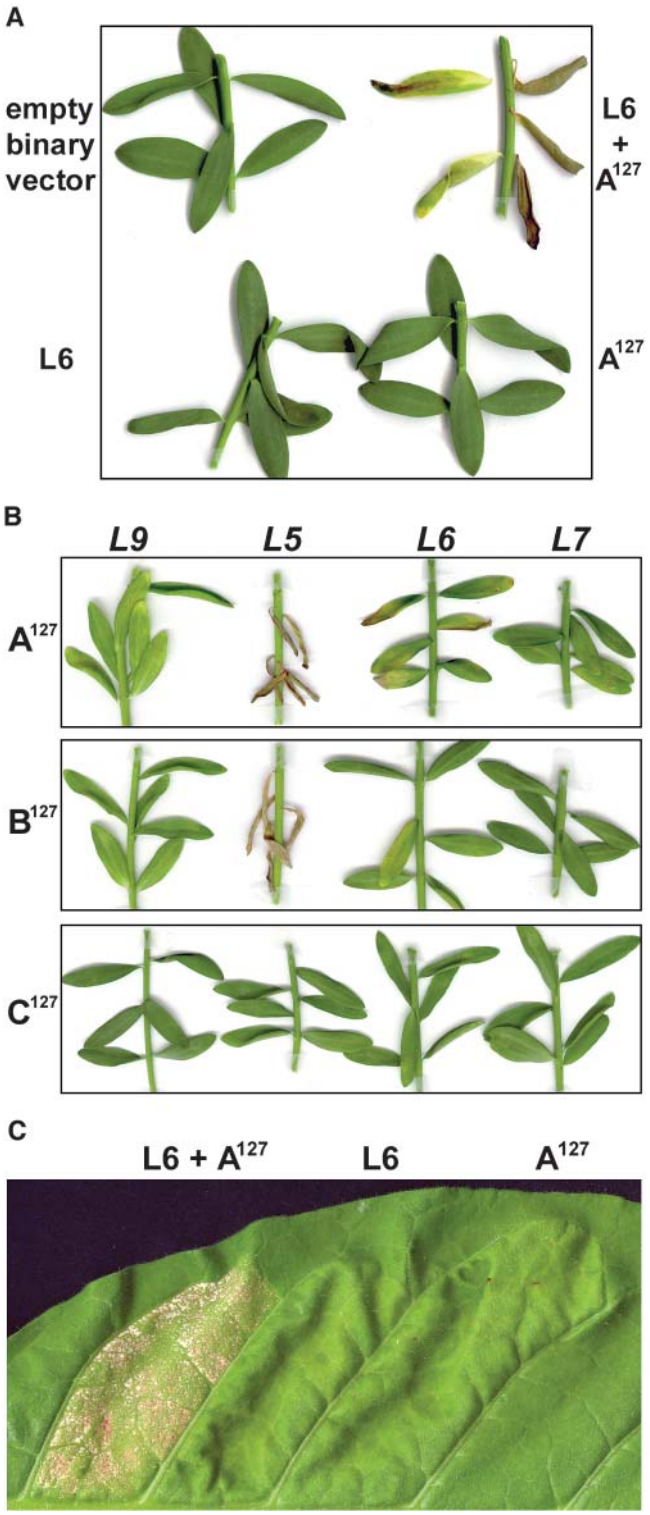


**Figure 3.** Transient Expression of *AvrL567* Genes in *L. usitatissimum* and *N. tabacum* Causes *R* Gene–Dependent Necrosis.

**(A)** Leaves of rust-susceptible *L. usitatissimum* var Hoshangabad were infiltrated with *A. tumefaciens* cultures containing an empty binary vector, an *L6* T-DNA vector, a T-DNA vector encoding AvrL567-A$^{127}$, or a mixture of two cultures containing the *L6* and AvrL567-A$^{127}$ T-DNAs.
**(B)** Leaves of near-isogenic *L. usitatissimum* lines containing the *L9*, *L5*, *L6*, or *L7* resistance gene were infiltrated with *A. tumefaciens* cultures containing T-DNA expression vectors encoding AvrL567-A$^{127}$, AvrL567-B$^{127}$, or AvrL567-C$^{127}$. Images were prepared 12 d after infiltration.
**(C)** W38 *N. tabacum* leaves were infiltrated with *A. tumefaciens* cultures containing either an *L6* T-DNA vector, a T-DNA vector encoding AvrL567-A$^{127}$, or a mixture of two cultures containing the *L6* and AvrL567-A$^{127}$ T-DNAs. Each *A. tumefaciens* culture was infiltrated into separate regions of the leaf bounded by the major veins, and the leaf was photographed 11 d after infiltration.

**Table 1.** Response of Near-Isogenic *L. usitatissimum* Lines to *Avr* Gene Transient Expression[a]

| | *L* Gene Allele Present in *L. usitatissimum* Lines | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene Construct | *L* | *L1* | *L2* | *L3* | *L4* | *L5* | *L6* | *L7* | *L8* | *L9* | *L10* |
| AvrL567-A$^{127}$ | – | – | – | – | – | + | + | +/– | – | – | – |
| AvrL567-B$^{127}$ | – | – | – | – | – | + | +/– | – | – | – | – |
| AvrL567-C$^{127}$ | – | – | – | – | – | – | – | – | – | – | – |

[a] Leaves were infiltrated with *A. tumefaciens* strains containing T-DNA constructs encoding the AvrL567$^{127}$ proteins and scored for the induction of necrotic responses 12 d after infiltration. A plus sign indicates necrosis observed, a minus sign indicates no necrosis, and a plus and minus sign together indicates a weak necrotic response.

Again this was dependent on the expression of both transgenes because transient expression of either L6 or AvrL567-A$^{127}$ alone did not produce a necrotic response. In a similar assay, co-expression of L6 and AvrL567-B$^{127}$ gave a weak response in *N. tabacum*, but no response was observed with coexpression of L6 and AvrL567-C$^{127}$ (data not shown). Similar results also were observed when the *AvrL567* genes were expressed in *N. tabacum* lines carrying an *L6* transgene (data not shown). Thus, the responses in *N. tabacum* are consistent with the recognition specificities observed in *L. usitatissimum*. Also, as observed in *L. usitatissimum*, constructs encoding the full-length AvrL567-A$^{150}$ and AvrL567-B$^{150}$ proteins, including the predicted signal peptides, produced much weaker responses than the truncated versions (data not shown).

### Coexpression of Resistance and Avirulence Genes in Transgenic Plants Causes Seedling Death

Specific HR induction also has been observed when stably transformed plants expressing Avr products are crossed to plants containing the corresponding *R* genes, with the progeny showing seedling death or stunted growth phenotypes (Jones et al., 1994; Gopalan et al., 1996; Hammond-Kosack et al., 1998; Erickson et al., 1999). We therefore generated transgenic plants containing the AvrL567$^{127}$ or AvrL567$^{150}$ construct in *L. usitatissimum* var Ward, which contains *L9*. No visible

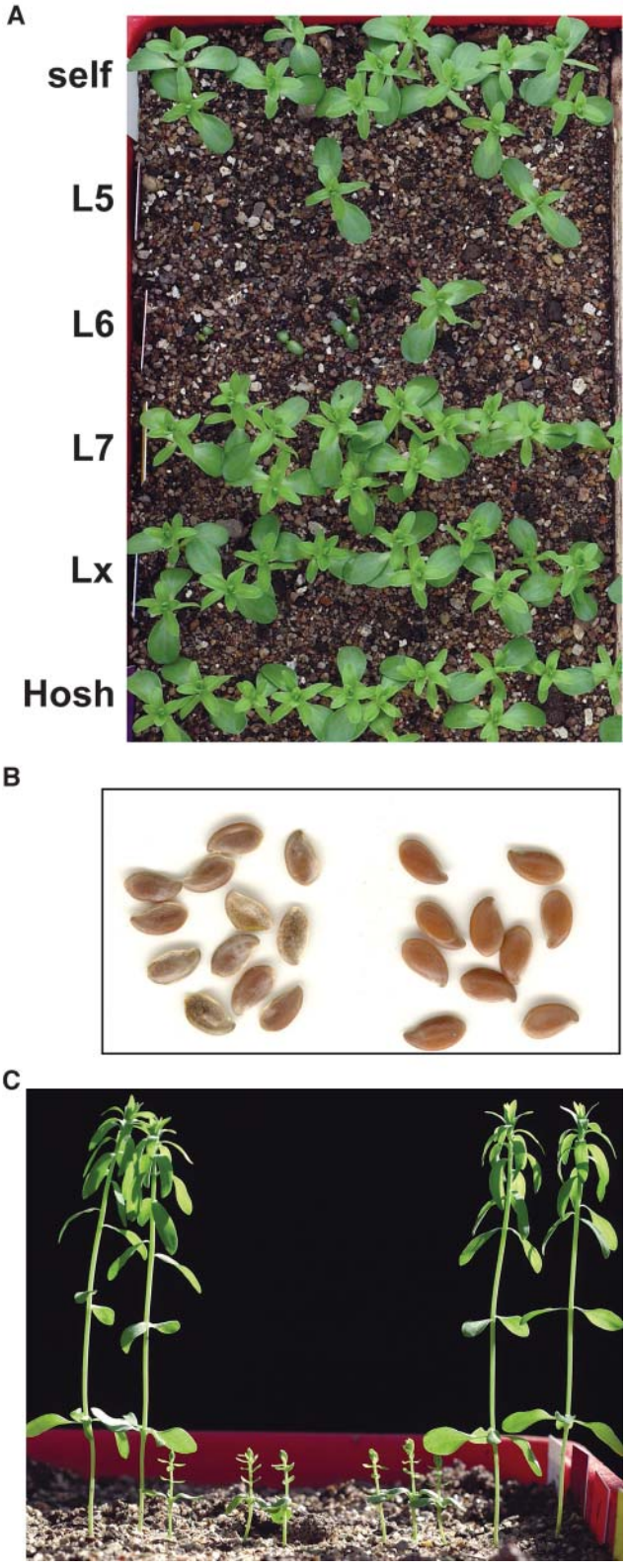


**Figure 4.** Phenotypes Resulting from Crossing AvrL567 Transgenes into *L. usitatissimum* Lines with Different *L* Genes.

phenotypes were observed among the primary T0 transgenic plants. For each construct, at least three independent T0 plants were crossed to lines homozygous for the *L5*, *L6*, *L7*, or *Lx* resistance gene (*Lx* has an identical specificity to *L7* but confers a stronger resistance phenotype; Luck et al., 2000). As a control, each T0 plant was crossed to the universally susceptible line Hoshangabad, and self-fertilized T1 seed were collected. All progeny from these control fertilizations showed normal growth (data not shown). However, progeny resulting from some crosses involving *L5*, *L6*, *L7*, or *Lx* segregated for seedling lethal or stunted growth phenotypes. For example, Figure 4A shows progeny resulting from crosses involving one T0 plant containing a single (hemizygous) copy of the AvrL567-$A^{150}$ transgene. No abnormal phenotypes were observed among the self-progeny or the outcross progeny involving the Hoshangabad, *L7*, or *Lx* parent. However, in the crosses to the *L5* or *L6* parent, the progeny segregate for wild-type and stunted phenotypes. Table 2 summarizes the seedling phenotypes observed in progeny of crosses between each of the T0 transgenic plants and the panel of resistant lines. The severity of these phenotypes varied between different outcross families but was consistent within each family. In the most severe cases, thin incompletely filled seeds were produced, and these failed to germinate when placed on wet filter paper (score = 0; Figure 4B). In other cases, apparently normal seeds were produced that germinated in vitro but gave rise to stunted seedlings. When germinated in soil, these seedlings either failed to emerge (score = 1; Figure 4A, *L5* cross), were arrested in growth after cotyledon emergence (score = 2; Figure 4A, *L6* cross), or gave rise to extremely stunted (score = 3; Figure 4C) or moderately stunted (score = 4; data not shown) dwarf plants. In some crosses, only wild-type progeny (score = 5) were observed. Segregation of wild-type and stunted phenotypes was generally consistent with the 1:1 ratio expected for single locus transgenes, although the sample sizes were small (12 seeds for each cross were assayed).

The specificity of the interactions observed in this experiment were very similar to those observed in the agroinfiltration transient expression assays (Figure 3, Table 1). Five independent T0 plants containing the AvrL567-$A^{127}$ transgene each gave rise to nonviable seed when crossed to the *L5*, *L6*, or *Lx* line. A milder

**(A)** A single T0 transgenic plant containing one copy of an AvrL567-$A^{150}$ transgene was crossed to *L. usitatissimum* lines homozygous for the *L5*, *L6*, *L7*, or *Lx* resistance gene as well as to the rust susceptible line Hoshangabad (Hosh). Twelve seeds from each cross as well as self-fertilized seeds were planted in soil. Photographs were taken 2 weeks after planting.

**(B)** Seeds resulting from a cross between a T0 transgenic plant containing one copy of the AvrL567-$A^{127}$ transgene and a *L. usitatissimum* line homozygous for *L5*. Approximately half of the seeds have a shrunken appearance (left) and are unable to germinate, whereas the phenotypically wild-type seeds (right) give rise to normal plants.

**(C)** Segregation of wild-type and dwarf phenotypes among 3-week-old seedlings resulting from a cross between a T0 transgenic plant containing one copy of the AvrL567-$B^{127}$ transgene and a *L. usitatissimum* line homozygous for *L6*.

**Table 2.** Abnormal Growth Phenotypes of Progeny from Crosses between *AvrL567*-Transformed *L. usitatissimum* and Various *L* Gene Lines

| Transgene | # T0 Plants[a] | Phenotypes[b] of Progeny from Crosses to: | | | |
|---|---|---|---|---|---|
| | | *L5* | *L6* | *L7* | *Lx* |
| AvrL567-A$^{127}$ | 5 | 0 | 0 | 3 to 4 | 0 |
| AvrL567-A$^{150}$ | 9 | 1 to 3 | 1 to 5 | 5 | 4 to 5 |
| AvrL567-B$^{127}$ | 7 | 0 to 3 | 2 to 5 | 5 | 5 |
| AvrL567-B$^{150}$ | 7 | 1 to 3 | 4 to 5 | 5 | 5 |
| AvrL567-C$^{127}$ | 3 | 5 | 5 | 5 | 5 |
| AvrL567-C$^{150}$ | 7 | 5 | 5 | 5 | 5 |

[a] The number of independent transgenic T0 plants used in crosses to *L* gene lines.

[b] Each T0 plant was crossed to the *L5*, *L6*, *L7*, and *Lx* lines. Each cross was scored according to the severity of any stunted growth phenotype segregating among the progeny. For each *AvrL567* transgene/*L* gene combination, the range of scores obtained for crosses involving independent T0 plants is shown. Numerical scores represent the following phenotypes: 0, incompletely filled seed, no germination in vitro; 1, seeds germinate in vitro, but seedlings do not emerge from soil; 2, seedling growth arrested after cotyledon emergence; 3, extreme dwarf seedlings; 4, moderate dwarf seedlings; and 5, all progeny have wild-type growth.

stunted phenotype was observed when these plants were crossed to the *L7* line (score = 3 to 4). Of seven T0 plants expressing AvrL567-B$^{127}$, four gave rise to nonviable seed when crossed to *L5*, whereas the remainder gave rise to extremely stunted progeny (one plant each with a score of 1, 2, or 3). Progeny of these seven T0 plants crossed to L6 showed a range of weaker phenotypes from seedlings arrested after cotyledon emergence (score = 2, one T0 plant) to dwarf seedlings (four T0 plants with a score of 3 and one T0 plant with a score of 4), and one T0 plant gave only wild-type progeny (score = 5). This weaker phenotype in the *L6* background compared with *L5* was consistently observed for each of the individual T0 plants, with seedling phenotype scores 2 to 3 points higher in the crosses to *L6* than in the crosses to *L5*. No effects were observed when the AvrL567-B$^{127}$ plants were crossed to *L7* or *Lx* (Table 2). DNA gel blot hybridization and/or PCR analysis of genomic DNA from individual progeny of several crosses showed that the AvrL567 transgene cosegregated with the abnormal growth phenotypes (data not shown).

Just as in the transient assays, constructs encoding the AvrL567-A$^{150}$ and AvrL567-B$^{150}$ full-length proteins showed weaker responses than the truncated versions but with a similar relative specificity (Table 2). For instance, nine independent AvrL567-A$^{150}$ T0 plants gave rise to progeny with a range of severe to mild stunted phenotypes when crossed to *L5* or *L6*, a very mild phenotype when crossed to *Lx*, and only wild-type progeny when crossed to *L7*. This weaker interaction was not because of lower transgene expression because we detected equivalent transcript levels in transgenic plants with the full-length and truncated constructs (data not shown). No abnormal phenotypes were observed in crosses involving AvrL567-C$^{127}$ or AvrL567-C$^{150}$, although DNA gel blot hybridization showed that these transgenes were inherited in crosses to the *L5* and *L6* lines. RNA gel blot analysis also showed that the AvrL567-C$^{150}$ construct was expressed at similar levels to the avirulence allele constructs in the transgenic plants. However, the three AvrL567-C$^{127}$ transgenic plants showed low expression of this transgene, so the lack of any response in these crosses is not conclusive.

### *AvrL567* Genes Are Expressed in Haustoria

We examined the expression pattern of the *AvrL567* genes in rust by RNA gel blot analysis (Figure 5A). *AvrL567* transcripts were detected in RNA samples derived from *L. usitatissimum* leaves infected with rust strain CH5 but not in RNA from germ tubes of rust spores germinated in vitro. This suggested that *AvrL567* expression was induced during infection. On the other hand, the linked *Sec14* and *orf-3* rust genes and an *M. lini* β-tubulin gene (Ayliffe et al., 2001) were expressed in germinated spores as well as during infection. *AvrL567* transcripts were detected in RNA from leaves infected with rust strains homozygous for either the virulence or avirulence allele (Figure 5A), indicating that the *AvrL567-C* gene is expressed in rust. The slightly lower amount of transcript detected from the virulent rust is consistent with the presence of only a single *AvrL567* gene at this allele compared with the two copies present at the avirulence allele. Because of the low levels of rust RNA present in infected leaves at early time points after infection, we used RT-PCR to examine *AvrL567* gene expression at these early stages (Figure 5B). Using this more sensitive assay, we still did not detect any *AvrL567* transcript in RNA from rust spores germinated in vitro, but it was detected in leaves 24 h after inoculation. Expression of the rust tubulin and *Sec14* genes also was detected by RT-PCR at 24 h after inoculation.

The HR induced by avirulent *M. lini* strains on *L. usitatissimum* plants containing *L6* first occurs in plant cells that contain a haustorium (Kobayashi et al., 1994), suggesting that avirulence genes are likely to be expressed in these structures. We used affinity chromatography with a Sepharose concanavalin A column (Hahn and Mendgen, 1992) to purify haustoria from *L. usitatissimum* leaves infected with rust CH5. The presence of intact haustoria in these samples was confirmed by microscopy and immunolabeling with a monoclonal antibody that detects polysaccharide epitopes present on the haustorial surface (Figure 5C; Murdoch et al., 1998). No contaminating plant cells or fungal mycelia were observed in the purified haustorial samples, although some chloroplasts were present. The *AvrL567* transcript was highly abundant in RNA isolated from the purified haustoria (Figure 5A). A cDNA library prepared from the purified *M. lini* haustorial RNA was screened with an *AvrL567* probe, which identified 4, 2, and 13 cDNA clones derived from *AvrL567-A*, *AvrL567-B*, and *AvrL567-C*, respectively, indicating that each of the three genes is expressed in haustoria.

### Evidence for Diversifying Selection at the *AvrL567* Locus

Because the three AvrL567 proteins were highly polymorphic (Figure 2B), we examined the patterns of nucleotide variation

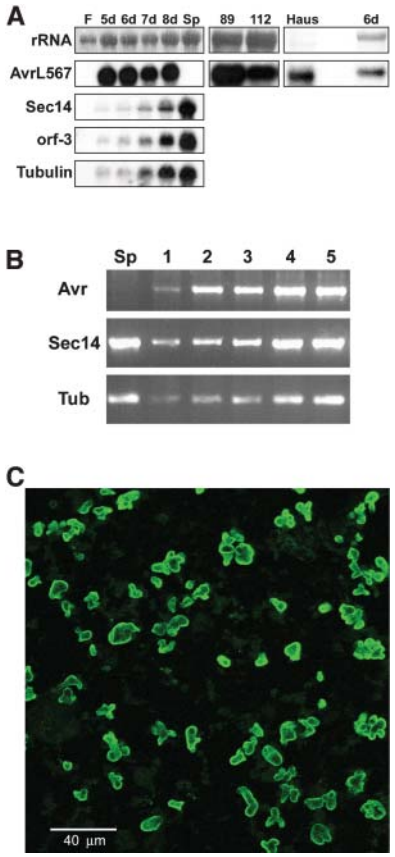


**Figure 5.** *AvrL567* Transcripts Are Induced during Infection and Are Expressed in Haustoria.

**(A)** RNA samples (5 μg) from uninfected *L. usitatissimum* leaves (F), rust CH5–infected *L. usitatissimum* leaves 5 to 8 d after infection (5d to 8d), and in vitro–germinated CH5 rust spores (Sp) were separated on a 1.5% agarose gel, transferred to nylon membranes, and hybridized with probes for the *AvrL567*, *Sec14*, *orf-3*, and tubulin genes of *M. lini*. Note that the infected leaf samples contain largely *L. usitatissimum* RNA with an increasing proportion of rust RNA as the fungal biomass increases during infection. The *AvrL567* probe also was hybridized to RNA from leaves infected with rust strain CH5-F2-89 (homozygous for the avirulence allele) or CH5-F2-112 (homozygous for the virulence allele). RNA extracted from 50 mg of haustoria purified from *L. usitatissimum* leaves 6 d after infection with rust CH5 (Haus) was run alongside 2.5 μg of RNA from rust CH5 infected leaves (6d) and hybridized to the AvrL567 probe. RNA filters were stained with methylene blue to detect rRNA loading (top panel). Given the low amount of haustorial RNA, the *AvrL567* signal in haustoria reflects a considerable enrichment compared with the infected leaf RNA.

**(B)** RNA from in vitro–germinated rust spores (Sp) and infected *L. usitatissimum* leaves after 1 to 5 d was reversed transcribed and amplified by PCR using primers specific for the *AvrL567* (Avr), *Sec14*, or tubulin (Tub) gene. RT-PCR products were separated on a 2% low melting point agarose gel and visualized by ethidium bromide staining and UV irradiation.

**(C)** Haustoria isolated from *L. usitatissimum* leaves 6 d after infection were fixed on glass slides and labeled with monoclonal antibody mL1 (Murdoch et al., 1998), followed by a goat anti-mouse secondary antibody coupled to the fluorophore Alexa-488 and visualized by fluorescence under illumination by 488-nm light.

at the *AvrL567* locus for evidence of positive selection. We aligned the 7357-bp DNA sequences surrounding each of the three *AvrL567* gene homologs, which correspond to the region duplicated in the *AvrL567-A* and *AvrL567-B* alleles (Figure 2A). The nucleotide variation in this region was highly concentrated within the 450-bp *AvrL567* coding sequence. A total of 30 single nucleotide differences, giving rise to 27 amino acid differences, occur in the coding region (average pairwise distance = 0.049). By contrast, only 25 single nucleotide changes occur in the 6907 bp of flanking sequence (average pairwise distance = 0.0022). This difference is highly significant (*t* test; $P < 0.001$) and suggests that selection has contributed to the accumulation of sequence differences within the coding sequences. There is also an excess of nucleotide changes at nonsynonymous sites (average distance = 0.061) over synonymous sites (average distance = 0.007) in the *AvrL567* genes (ratio = 8.6). A G-test showed that this difference was significant for the pairwise comparisons between *AvrL567-A* and *AvrL567-B* ($P < 0.04$; 19 substitutions in 341 nonsynonymous sites and 1 substitution in 109 synonymous sites) and between *AvrL567-A* and *AvrL567-C* ($P < 0.002$; 27/341 and 0/109) but not between *AvrL567-B* and *AvrL567-C* ($P < 0.08$; 15/342 and 1/108). (Zhang et al., 1997 recommended a G-test for significance when there are fewer than 10 substitutions at one class of site because the *t* test overestimates the significance value under these conditions.) This indicates that selection has favored the accumulation of amino acid variation in these genes (Hughes and Nei, 1988).

The only other significant variation between these sequences occurs in the *AvrL567* promoter region. Each gene contains either 12 (*AvrL567-A*) or 13 (*AvrL567-B* and *AvrL567-C*) copies of an 82-bp repeated sequence (Figure 2A). The last of these repeats ends ~150 bp from the putative transcription start site. There is also one 13-bp insertion/deletion polymorphism that occurs 750 bp downstream of the AvrL567 coding sequence. The DNA sequences flanking the 7357-bp duplication also are highly conserved between the two alleles, with only 10 nucleotide differences in the 3200-bp 5′ region and five differences in the 2460-bp 3′ region (distance = 0.0026). The only major difference is the insertion of a 6270-bp sequence in the 5′ flanking region of the avirulence allele (Figure 2A), which includes 173-bp identical direct repeats and contains an open reading frame that is closely related to gypsy class retrotransposon pol protein sequences from *Tricholoma matsutake*, *Magnaporthe grisea*, and *Fusarium oxysporum*.

## DISCUSSION

We have isolated genes previously from four major rust resistance loci in *L. usitatissimum* (Lawrence et al., 1995; Anderson et al., 1997; Dodds et al., 2001a, 2001b), but the corresponding avirulence genes in *M. lini* had not been identified. We now have identified *M. lini* avirulence genes corresponding to the *L5*, *L6*, and *L7* alleles of the *L* resistance locus in *L. usitatissimum*. The *AvrL567-A* and *AvrL567-B* genes cosegregate with the avirulence phenotype in an F2 family of 74 individuals and cause *R* gene–dependent cell death when expressed in transiently transformed leaves of *L. usitatissimum* (Figures 3A

and 3B, Table 1) and *N. tabacum* (Figure 3C). Induction of cell death occurred when the *AvrL567* genes were coexpressed with *L5*, *L6*, or *L7* but not other *L* locus resistance genes. Importantly, the *AvrL567-C* gene, which segregates with the virulence allele, did not cause necrosis with any of the *L* resistance genes. In addition, a stunted growth phenotype was observed when the corresponding *R* and *Avr* genes were brought together by crossing transgenic *L. usitatissimum* expressing *AvrL567* genes to resistant lines (Figure 4, Table 2). Although we have not been able to directly test the phenotype conferred by these genes in rust, these results provide compelling evidence that the *AvrL567-A* and *AvrL567-B* genes are responsible for recognition by the *L5*, *L6*, and *L7* resistance genes.

### AvrL567 Proteins Are Secreted from Rust Haustoria and Recognized in Plant Cells

Haustoria are the primary site of contact between rust pathogens and host mesophyll cells. Rust infection involves germination of the rust spore, growth of the germ tube on the leaf surface, and then appresorium formation over a stoma to allow penetration of the leaf. Inside the leaf, a substomatal vesicle forms and growth of infection hyphae leads to differentiation of a haustorial mother cell that extends a haustorium into a leaf mesophyll cell. In rust-infected *L. usitatissimum*, the first haustoria appear ~12 h after infection, and by 24 h, haustoria have formed at almost all penetration sites (Kobayashi et al., 1994). In *L6*-mediated resistance, hypersensitive cell death is induced in cells containing developing haustoria by 24 h after infection (Kobayashi et al., 1994). The *AvrL567* transcripts were not detected in germ tubes produced by in vitro germinated rust spores but were induced during infection within 24 h (Figures 5A and 5B). The relative enrichment of the *AvrL567* transcript in the isolated haustoria samples (Figure 5A) suggests that these genes may be predominantly expressed in haustoria, although we do not know whether there is expression in other rust cell types present in infected leaves. These observations are consistent with the timing and location of HR induction in resistant *L6* plants.

The AvrL567 protein sequences contain predicted signal peptides (Figure 2B), which can direct secretion of a reporter protein in yeast (A.-M. Catanzariti, unpublished results). Thus, we predict that these avirulence products are secreted from *M. lini* haustoria into the surrounding extrahaustorial matrix. In planta expression of the AvrL567-A$^{127}$ and AvrL567-B$^{127}$ proteins, which lack the signal peptide, induced strong necrotic responses in conjunction with the corresponding *L* resistance genes (Figures 3 and 4, Tables 1 and 2). Thus, recognition of the AvrL567 products occurs when they are expressed within plant cells, which is consistent with the predicted location of the L resistance proteins. However, intracellular recognition during infection would require translocation of the secreted AvrL567 proteins across the plant plasma membrane, which separates the extrahaustorial matrix from the host cytoplasm. Similar observations for bacterial Avr proteins provided the first indication that these products were translocated directly into plant cells (Gopalan et al., 1996; Leister et al., 1996; Scofield et al., 1996; Tang et al., 1996; van den Ackervecken et al., 1996). Other known rust resistance genes in *L. usitatissimum* (Lawrence et al., 1995; Anderson et al., 1997; Dodds et al., 2001a, 2001b), maize (Collins et al., 1999), and *H. vulgare* (Brueggeman et al., 2002) also encode predicted cytoplasmic proteins, so it is possible that a variety of rust Avr products are targeted to the host cytoplasm. The AvrL567 proteins may represent a class of rust effector proteins that are translocated into plant cells to facilitate infection during a compatible infection.

No specific translocation mechanism to direct effector proteins into host cells has been described in fungal pathogens. Although Avr products identified in other plant pathogenic fungi also are secreted proteins (Lauge and de Wit, 1998), the Avr2, Avr4, and Avr9 products of *Cladosporium fulvum* (Parniske et al., 1997; Luderer et al., 2002) and the *Rhyncosporium secalis* NIP1 protein (Knogge, 1996) are recognized extracellularly and probably do not enter plant cells. Delivery of the Avr1b protein from the oomycete pathogen *Phytophthora sojae* to the extracellular space of *Glycine max* (soybean) leaves induced *R* gene–dependent cell death (Tyler 2002), suggesting that it also may be recognized extracellularly. On the other hand, the *M. grisea*–secreted protein AVR-PITA is recognized via a direct interaction with a putatively cytoplasmic R protein from rice (*Oryza sativa*) (Jia et al., 2000). However, infection hyphae of *M. grisea* penetrate plant cells directly, and no host membrane separating the fungus from the plant cytoplasm has been described.

### AvrL567 Proteins Have Overlapping Recognition Specificities

The cosegregation of avirulence on *L5*, *L6*, and *L7* has suggested that multiple avirulence genes may occur at this locus. However, although there are two related avirulence genes at this locus in the CH5-F2 family, these genes show overlapping recognition specificities. *AvrL567-A* is recognized by all three resistance genes, whereas *AvrL567-B* is recognized most strongly by *L5* but only weakly by *L6* and not at all by *L7* (Tables 1 and 2, Figure 3B). Similar overlapping recognition specificities have been observed in other gene-for-gene resistance systems. For instance, the RPM1 resistance protein in Arabidopsis recognizes two unrelated avirulence products from *P. syringae*, AvrRpm1 and AvrB (Grant et al., 1995), and these two Avr proteins are recognized by distinct *R* genes in *G. max* (Ashfield et al., 1995).

We speculated previously that the L6 and L7 resistance proteins may recognize the same or very similar avirulence products because there are only 11 amino acid differences between these proteins, all in the N-terminal TIR domain, and just three of these changes, in the recombinant Lx protein, are sufficient to alter L6 to express the L7 specificity (Ellis et al., 1999; Luck et al., 2000). Furthermore, these specificities are only distinguished by a separate inhibitor gene (*I*) from *M. lini*, which prevents L7- and Lx-mediated resistance but has no effect on L6-mediated resistance (Lawrence et al., 1981). The difference between the *L6* and *L7* specificities in our assays with the *AvrL567* genes seems to be largely one of degree. *L6* interacts strongly with *AvrL567-A* but weakly with *AvrL567-B*, whereas *L7* interacts weakly with *AvrL567-A* but not with *AvrL567-B* (Tables 1 and 2, Figure 3B). This is consistent with the weaker resistance

phenotype conferred by *L7*, which allows the formation of small pustules, relative to *L6*, which completely restricts rust growth (Islam and Mayo, 1990). The recombinant *Lx* gene gives a strong resistance phenotype in infected plants and also gives stronger recognition of AvrL567-A in transgenic plants than *L7* (Table 2) but is still completely inhibited by the *I* gene. It is not clear how the *I* gene product may inhibit L7 and Lx resistance, although it apparently does not affect the expression of *AvrL567* genes because similar transcript levels accumulate in rust strains with or without *I* (P.N. Dodds, unpublished results). One intriguing possibility is that the *I* gene product may interfere directly with the L7 and Lx proteins through their TIR domains to block signaling. Alternatively, the AvrL567 products may be altered in a way that prevents recognition by L7 and Lx but not L6.

The overlap in recognition properties between L5 and L6 was more surprising because there are 104 amino acid differences between these proteins, which are among the most highly diverged of the L protein family. There are seven other rust resistance specificities encoded by the known alleles of the *L. usitatissimum L* gene, and the corresponding *Avr* genes in *M. lini* map to seven independent loci. However, hybridization of *AvrL567* probes to *M. lini* genomic DNA did not detect any related sequences even at low stringency (data not shown). Therefore, despite the high sequence similarity of these *L* gene alleles, the corresponding *Avr* genes are not closely related to the *AvrL567* genes. The overlap between the *L5* and *L6* recognition specificities may be a result of convergent evolution. Although all of the rusts in our collection show the same reaction on both *L5* and *L6*, Flor (1955) described a rust strain that was avirulent on plants containing *L6* but virulent on *L5* plants. Thus, some rust strains may carry variants of *AvrL567* that can distinguish between *L5* and *L6*. The weaker recognition of *AvrL567-B* by *L6* compared with *L5* in our expression assays provides some evidence for differential recognition. In fact, one *AvrL567* gene variant, isolated from a rust strain unrelated to strains C and H, is recognized by *L6* but not *L5* in the transient expression assay (P.N. Dodds, unpublished results).

Another question we have considered is how the virulent rust strains carrying *AvrL567-C* escape recognition. This does not appear to be because of differences in gene expression because *AvrL567* transcripts were observed at similar levels in both virulent and avirulent rust strains (Figure 5A), and a cDNA library from rust CH5 haustoria contained representatives of all three *AvrL567* genes. The lack of any significant sequence divergence outside the coding sequences of the *AvrL567* genes also would argue against differential expression of these genes. The *AvrL567-C* expression constructs in our transient expression assays were identical to the *AvrL567-A* and *AvrL567-B* constructs outside of the coding sequence and generated similar amounts of mRNA. Thus, the lack of recognition of *AvrL567-C* in these assays suggests that the virulence phenotype is conferred by the differences in the coding sequence rather than differences in gene expression or protein modifications occurring in the rust. There are 10 polymorphic amino acids unique to the AvrL567-C protein (Figure 2B) that may explain its lack of recognition by any known *R* gene in *L. usitatissimum*. One possibility is that these changes may result in reduced protein stability. However, both the AvrL567-A and AvrL567-C proteins can accumulate to high levels in *Escherichia coli* when expressed with a 6xHis tag (A.-M. Catanzariti, unpublished results). An alternative possibility is that the AvrL567-C protein is stable, but the amino acid changes interfere with recognition by the resistance proteins. In this scenario, AvrL567-C may retain the presumed pathogenicity function performed by this family of proteins in *M. lini*.

### Adaptive Evolution of the *AvrL567* Genes

Evolution of the *AvrL567* genes has been characterized by strong positive selection for amino acid variation between the encoded products. This is shown by the significant excess of nucleotide variation within the AvrL567 coding sequence relative to the flanking DNA as well as the evidence for diversifying selection acting in these genes. This could be explained by selective pressure for the pathogen to avoid recognition-triggered resistance in the host and is consistent with our interpretation that the lack of AvrL567-C recognition is attributable to the amino acid differences from AvrL567-A and AvrL567-B. The observation that diversifying selection acts on the *AvrL567* genes as well as on the *L. usitatissimum L* genes (Dodds et al., 2000) is consistent with an arms race model of coevolution between *R* and *Avr* genes driven by selection for resistance in the host and virulence in the pathogen (Bergelson et al., 2001). This system provides the first example in which evidence for such adaptive evolution has been found in both components of an *R*/*Avr* gene pair. Although *R* genes impose an obvious selective pressure on pathogen *Avr* genes and host resistance strongly influences pathogen virulence in natural populations of *M. lini* on the wild flax species *L. marginale* (Thrall and Burdon, 2003), it is possible that other factors have driven the *AvrL567* gene diversification. For instance, if, like some bacterial Avr proteins (Lahaye and Bonas, 2001; Axtell et al., 2003; Mackey et al., 2003; Shao et al., 2003), the AvrL567 proteins have an effector role in rust infection that involves targeting a specific host protein(s), an alternative arms race independent of *R* genes could occur between *AvrL567* and the host gene(s) encoding this target. In this case, variation in the host gene that prevents targeting may select for changes in the pathogen *Avr* gene to re-engage the host target.

### Conservation of L6/AvrL567-A Recognition and HR Induction in *N. tabacum*

Many resistance proteins have been shown to function in heterologous plant species to trigger a defense response that is dependent on their corresponding Avr products (Thilmony et al., 1995; Whitham et al., 1996; Hammond-Kosack et al., 1998; Tai et al., 1999; van der Hoorn et al., 2000). However, most of these examples involve R proteins transferred to closely related species, and transfer to species outside the original family has generally not been successful, with a few exceptions. For instance, the *L. esculentum* Cf resistance proteins can function in some more distantly related species because Cf-4/Avr4 recognition induces an HR in *L. sativa* (van der Hoorn et al., 2000), and Cf-9/Avr9 recognition induces an HR in Brassica (Hennin et al., 2001). The Arabidopsis *RPW8.1* and *RPW8.2* powdery mildew resistance genes also confer resistance to

powdery mildew pathogens when expressed in *N. tabacum* (Xiao et al., 2003). We have found that the recognition interaction between the L6 and AvrL567 proteins can occur in *N. tabacum* cells and results in HR induction. Thus, if any other host proteins are involved in this recognition interaction as suggested by the guard hypothesis (Dangl and Jones, 2001), then these proteins must be sufficiently conserved between *L. usitatissimum* and *N. tabacum* to support the same recognition events. Alternatively, the L6 and AvrL567 products may interact directly. Furthermore, this observation indicates that the L6 protein can interact productively with the *N. tabacum* resistance-signaling pathway.

## METHODS

### Rust and Plant Material

An *M. lini* F2 family derived from selfing rust strain CH5, the F1 hybrid of a cross between parental strains C and H, was described by Lawrence et al. (1981). Near-isogenic *L. usitatissimum* lines containing the *L*, *L1*, *L2*, *L3*, *L4*, *L5*, *L6*, *L7*, *L8*, or *L10* allele backcrossed for 6 to 12 generations into *L. usitatissimum* var Bison, which contains *L9*, were described by Flor (1954). Rust inoculations were performed as described by Lawrence et al. (1981). Rust spores were germinated overnight on water, and genomic DNA was isolated from the resulting mycelial mat as described (Anderson et al., 1997).

### Isolation of Rust cDNAs

RNA was isolated from leaves of rust-susceptible *L. usitatissimum* var Hoshangabad 6 d after inoculation with urediospores of rust strain C or H or a mock treatment. Poly(A)$^+$ RNA was isolated from total RNA using a PolyAtract kit (Promega, Madison, WI) according to the manufacturer's instructions. Double-stranded cDNA was synthesized using a Superscript Choice cDNA synthesis kit (Gibco BRL, Rockville, MD). Subtracted cDNA libraries enriched for cDNAs more abundant in the tester than driver samples were prepared using suppression subtractive hybridization as described by Diatchenko et al. (1996) using an Advantage cDNA polymerase kit (Clontech Laboratories, Palo Alto, CA) and cloned into pGEMT-Easy (Promega). For the infection-specific library, the tester cDNA was prepared from leaves infected with rust stain H for 6 d, and the driver was from uninfected *L. usitatissimum* leaves. For the differential library, the driver was prepared from leaves infected with rust strain C for 6 d.

### Gel Blot Analysis

Restriction enzyme–digested genomic DNA was separated on 1.0% agarose gels and transferred to Hybond N+ nylon membranes (Amersham, Buckinghamshire, UK). RNA samples were separated on 1.5% agarose gels and transferred to Hybond N+. Prehybridization and hybridization with $^{32}$P-dCTP–labeled DNA probes were performed in 7% (w/v) SDS, 1% (w/v) BSA, 0.5 M sodium phosphate, pH 7.2, and 1 mM EDTA at 65°C, and washing was in 1× SSC and 0.1% SDS at 65°C.

### Isolation of Genomic Sequences

Lambda clones hybridizing to the IU2F2 cDNA probe were isolated from a λEMBL3 genomic DNA library prepared from rust strain CH5 (Ayliffe et al., 2001). A sequence contig of 26.5 kb was assembled by shotgun cloning and direct sequencing of overlapping λ clones. A second contig of 11 kb was assembled by direct sequencing of PCR products amplified from the F2 rust CH5F2-112 (homozygous for the virulence allele). The Perkin-Elmer/Applied Biosystems GeneAmp XL PCR kit was used (Roche Molecular Systems, Branchburg, NJ) with thermal cycling conditions as follows: 94°C for 2 min, 40 cycles of 94°C for 20 s, 55°C for 30 s, 72°C for 5 min, and then a final 10 min at 72°C. The primer pairs 10-1.8 (5′-TGCCTTGAGCCGGTGATTC-3′) and 10-1.16 (5′-TAATCCTCGTTGACATCAGTC-3′), 10-1.15 (5′-AAGCTTGAGAGCTCCGCTC-3′) and 10-1.9 (5′-GTCTCTTCGTCCTTCCAAG-3′), and 10-1.4 (5′-GTCGACCGATCTATATCGAG-3′) and 10-1.37 (5′-CGAGCATTTGTAGGCATTTGTC-3′) were used to generate overlapping PCR fragments of 4.5, 3.3, and 4.5 kb, respectively. These were purified using the QIAquick PCR purification kit (Qiagen, Clifton Hill, Australia) and sequenced directly. An additional 500-bp region at the 3′ end of this contig was identified by inverse PCR. Genomic DNA from the rust CH5F2-78 (homozygous for the virulent allele) was digested with EcoRV (MBI Fermentas, Vilnius, Lithuania) and religated with T4 DNA ligase (2 units; MBI Fermentas). The circularized DNA was amplified by PCR (94°C for 2 min, 40 cycles of 94°C for 20 s, 55°C for 30 s, 72°C for 2 min, and 72°C for 10 min) with the primer pairs 10-1.16 and 10-1.40 (5′-AGCGATTATAAAATGAGACAAG-3′) and then reamplified in a nested PCR with the primers 10-1.6 (5′-TTGCATGGATCCCACCAAG-3′) and 10-1.39 (5′-CACTGGAATTGATTTCAATCAC-3′). An amplified fragment of ~800 bp was cloned into pGEM T-Easy (Promega), and four independent clones were sequenced.

### Transcript Analysis by RT-PCR

Total RNA was reverse transcribed using Superscript reverse transcriptase (Gibco BRL) with an oligo(dT)$_{25}$ primer and then amplified by Taq polymerase with the following thermal profile: 94°C for 2 min, 38 cycles of 94°C for 20 s, 55°C for 30 s, 72°C for 1 min, and 72°C for 5 min. *AvrL567* transcripts were amplified with the primers 10-1.15 and 10-1.16, *Sec14* transcripts with 10-1.4 and 10-1.9, and *M. lini* tubulin transcripts with tub10 (5′-AAACACTAAATCAAACATGAGGG-3′) and tub12 (5′-ACAAAGAACCAAAAGGACCCGA-3′). Each primer set spans one or more introns to distinguish cDNA and genomic DNA derived products. *AvrL567* RT-PCR products from rust H–infected leaves were cloned into pGEMT-Easy, and 21 independent clones were sequenced to confirm the predicted coding regions and splicing sites.

### Transient Expression Assays

Gene expression constructs contained the *AvrL567* coding sequences inserted between a 35S promoter of *Cauliflower mosaic virus* and a nopaline synthase terminator in the binary vector pTNotTReg (Anderson et al., 1997). Constructs encoding the truncated AvrL567$^{127}$ proteins, lacking the signal peptide, begin at a BamHI site at position 59 relative to the first ATG, have an A-to-T alteration at position 65 to remove an out-of-frame ATG codon, and include 45 nucleotides 3′ to the stop codon. The open reading frame starts at Met-24, the first amino acid of the expected mature AvrL567 proteins. Constructs encoding the full-length AvrL567$^{150}$ proteins contain the entire coding sequences as well as 105 nucleotides 5′ to the first ATG. All constructs were fully sequenced to confirm their integrity. *A. tumefaciens* strains GV3101-pMP90 or C58C1 (which lacks the Ti plasmid) containing the binary vector expression constructs were prepared at an OD$_{600}$ of 1.0 in liquid MS medium containing 200 μM acetosyringone and infiltrated into *L. usitatissimum* or *N. tabacum* leaves. For coinfiltration, equal volumes of two *A. tumefaciens* cultures containing the *AvrL567* gene constructs (at OD$_{600}$ = 1.0) or an *L6* gene construct (Lawrence et al., 1995; at OD$_{600}$ = 1.0 for *L. usitatissimum* or 0.5 for *N. tabacum*) were mixed together before infiltration. For control infiltrations of the *AvrL567* or *L6* constructs alone, these cultures were mixed with an *A. tumefaciens* culture containing the empty binary vector in similar proportions.

### *L. usitatissimum* Transformation and Crosses

Transformation of the *L. usitatissimum* line Ward was as described by Anderson et al. (1997), except that the selective agent spectinomycin was used at 50 μg/mL. Genomic DNA was extracted from T0 plants using DNAzol reagent (Molecular Research Center, Cincinnati, OH) and analyzed by gel blot hybridization to determine transgene copy number. Selected plants containing one to three intact copies of the transgene were used as the female parents in crosses to *L. usitatissimum* lines carrying various *L* resistance genes. Flowers were emasculated before maturity and then hand pollinated. Twelve seeds from each cross were planted in soil to examine the phenotypes of the resultant progeny. Seeds from some crosses also were germinated in Petri dishes on wet filter paper.

### Isolation of Haustoria

Haustoria were isolated from *L. usitatissimum* leaves 6 d after inoculation with rust strain CH5 by affinity chromatography as described by Hahn and Mendgen (1992), except that an 11-μM pore size nylon mesh was used to remove plant cell material from the crude preparation. An affinity column was prepared by covalently attaching concanavalin A (Pharmacia Biotech, Uppsala, Sweden) to cyanogen bromide–activated Sepharose 6MB (Pharmacia Biotech) as described in the manufacturer's protocol. Samples of purified haustoria were examined by immunofluorescence labeling with monoclonal antibody mL1 as described by Murdoch et al. (1998), except that samples were fixed to slides in 0.5% gelatin, 0.05% chromic potassium sulfate, and 0.02% $NaN_3$, and the secondary antibody used was the goat anti-mouse coupled to the fluorophore Alexa 488 (Molecular Probes, Eugene, OR). Slides were examined on a Leica TCS SP2 confocal microscope (Leica, Mannheim, Germany) under light or under excitation by an argon ion laser at a wavelength of 488 nm. Images were collected at 500 to 530 nm with pseudocolor green. RNA was prepared from purified haustoria using the Qiagen Plant RNeasy kit. A cDNA library was prepared from 2 μg total RNA from haustoria using the SMART cDNA library kit (Clontech) according to the manufacturer's instructions.

### Sequence Analysis

The *AvrL567* coding sequence or flanking sequences were aligned, and nucleotide sequence distances were calculated across all sites or specifically for nonsynonymous and synonymous sites using the Jukes-Cantor algorithm of the Molecular Evolutionary Genetics Analysis software version 1.02 (Kumar et al., 1993). The significance of differences between average pairwise nucleotide distances was assessed by a *t* test, whereas a G-test was used to test the significance of differences in the number of nucleotide changes at nonsynonymous and synonymous sites in individual pairwise comparisons.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY510102 and AY510103.

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